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RAPID COMMUNICATION

Membrane Fusion between Retroviral Particles: Host-Range Extension and Vaccine Prospects

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We have analyzed if different populations of retroviral particles carrying the viral and cellular receptors of membrane viruses, respectively, are able to specifically fuse with each other. Using the glycoprotein of human immunodeficiency virus type 1 and its cellular receptor complex, we demonstrate that interviral membrane fusion can, indeed, occur and that the resultant fused viral structures are able to infect cells and transduce a marker gene. On the one hand, these results have relevance for the development of vaccine strategies based on fusion-induced conformational epitopes on the viral glycoprotein. However, in addition to this potential practical application, the results obtained (which were extended to include analyses with the vesicular stomatitis virus G protein and its cellular receptor) have far-reaching implications for *in vivo* situations in which simultaneous infections with different membrane viruses can occur.

Key Words: interviral fusion; host range; human immunodeficiency virus; HIV glycoprotein; vaccine; CD4; CXCR-4; cellular receptor; VSV-G; transduction.

Introduction. A putative "fusion-competent" whole-cell immunogen with the potential of eliciting broadly neutralizing HIV antibodies in CD4/CCR5 transgenic mice has recently been described (*1*). The relevant immunogen is presumed to consist of HIV Env conformations transiently arising during fusion between individual cells expressing CD4/coreceptor and HIV Env, respectively. It has previously been demonstrated that cellular glycoproteins can efficiently be incorporated into the membrane of retroviral and rhabdoviral particles and can function there to mediate infection of cells carrying the appropriate viral receptor (*2–5*). We thus considered the possibility that a more potent and cleaner fusion-competent HIV immunogen may be generated by transient fusion between two different types of subviral HIV-like particles with incorporated CD4/coreceptor and HIV Env, respectively. If this were the case, it was of further interest to establish if fused viral structures, carrying more than one core, were infectious (that is able to transduce a marker gene to target cells). In order to potentially achieve higher infectivity of fused viral structures, we extended these analyses to include the glycoprotein of vesicular stomatitis virus (VSV-G), which is a potent mediator of retroviral transduction (*6, 7*). We demonstrate here that specific interretroviral particle membrane fusion can occur, that fused retroviral particles are infectious (with increased infectivity mediated by VSV-G), and that these have the host range of both fusion partners. On the one hand, these results demonstrate the potential feasibility of an HIV vaccine strategy employing fixed preparations of molecularly well-defined HIV-like particles in the process of membrane fusion. Importantly, potential interviral fusion *in vivo* (e.g., in patients infected with two enveloped viruses) may have far-reaching consequences relevant for disease development and progress.

Results and Discussion. The first strategy which we employed to analyze membrane fusion between HIV-like particles is illustrated in Fig. 1A. Particles, with incorporated CD4 and CXCR-4 and carrying a transducible luciferase reporter gene, are referred to as *CD4/CXCR-4 target particles.* Particles with incorporated Wt HIV Env, but lacking transducible vector, are referred to as *Wt-Env fusion particles.* We hypothesized that if membrane fusion between target and fusion particles would occur, the resultant fused viral structures could be infectious for, and be able to transduce the luciferase gene to, CD4/CXCR-4-expressing 293T cells.

Target particles were generated by cotransfecting 293T cells with an Env-defective proviral construct, pNL4-3.Luc.R-E- (*8*), encoding the luciferase gene instead of *nef,* plus expression vectors for CD4 and CXCR-4, either alone or in combination. It has previously been shown that CD4 and CXCR-4 can be incorporated into HIV particles and mediate infectivity into HIV-1-infected cells (*5*) and we confirmed that our CD4/CXCR-4

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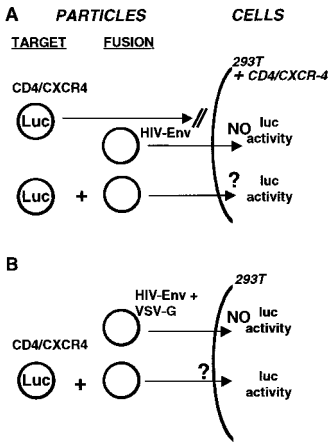

FIG. 1. Strategies to analyze interretroviral particle fusion and the infectivities of fused viral structures. (A) Target particles (left), generated as described under Materials and Methods, carry a genome encoding a marker luciferase gene (Luc) and have incorporated CD4 and CXCR-4. Fusion particles (middle) lack marker gene and have incorporated HIV Env. Target particles, fusion particles, or mixtures of target particles and fusion particles were analyzed as to their ability to transmit luciferase (luc) activity to indicator CD4/CXCR-4-expressing 293T cells. (B) As in A, but fusion particles have incorporated VSV-G in addition to HIV Env. The indicator cells are 293T cells, not expressing CD4/CXCR-4.

target particles were infectious for, and efficiently transmitted luciferase activity into, HIV Env-expressing BJAB-Env*i* cells (*9*) (data not shown). In the strategy shown in Fig. 1A, Wt-Env fusion particles, or mutants thereof, were generated by coexpressing an Env-defective proviral plasmid, with a deletion in the packaging signal, with Wt or mutant HIV Env. Appropriate volumes of culture supernatants, representing equal amounts of target particles on the one hand and equal amounts of fusion particles on the other hand, were mixed (at a ratio of 1:1) and preincubated for 90 min at 37°C to allow potential fusion to occur (see Materials and Methods). Mixed supernatants were subsequently applied to CD4/CXCR-4-expressing 293T cells and 3 days posttransduction, the luciferase activities in cell lysates were determined.

As shown in Fig. 2A, CD4/CXCR-4 target particles alone resulted in only very low levels of luciferase activity after transfer to CD4/CXCR-4-expressing 293T cells. However, when these CD4/CXCR-4 target particles were mixed with culture supernatants containing Wt-Env fusion particles, the resultant mixture led to a 30- to 50-fold increase in transduction of CD4/CXCR-4-expressing 293T cells. No transduction was observed employing 293T cells lacking CD4/CXCR-4. The luciferase values obtained were, in fact, about 2% of those obtained when Wt HIV Env itself, instead of CD4/CXCR-4, was directly incorporated into target particles (Fig. 2A). We consider this value of 2% to be relatively high. This is because, on the one hand, the particle concentration in the culture supernatants is in the range of only 1–5 μg/ml, making it unlikely that every target particle can fuse with a fusion particle. Additionally, it is unlikely that fused viral structures are as efficient in mediating luciferase gene transduction as genuine viral particles. Several controls were performed in order to confirm that the observed transduction was due to a fusion event (and not just a binding event) requiring all components, namely CD4, CXCR-4, and Wt HIV Env in a functional form. On the one hand, CD4 target particles or CXCR-4 target particles, with incorporated CD4 or CXCR-4 alone, respectively, were mixed with Wt-Env fusion particles. On the other hand, Env^{Fus-} fusion particles, with incorporated nonfusogenic HIV Env with a mutation in the gp41 fusion peptide, were mixed with CD4/CXCR-4 target particles. All these mixtures gave only background levels of transduction. These results clearly indicate that specific CD4/CXCR-4/HIV-Env-dependent interretroviral fusion can occur and that the resultant fused particles are infectious.

When we initially developed the experimental strategy illustrated in Fig. 1A, we had considered the possibility that the potential transduction by fused particles could be an inefficient process, and thus difficult to measure, since a fraction of the HIV Env would already have engaged in membrane fusion with the target particles and thus would no longer be available to mediate infection of cells. Although, as shown in Fig. 2A, this consideration was unwarranted, we concomitantly developed the alternative strategy illustrated in Fig. 1B, which would allow us to circumvent this potential problem. In this strategy, in addition to HIV Env, the fusion particles (now referred to as Wt-Env/G fusion particles) have incorporated VSV-G glycoprotein, which is a potent mediator of retroviral vector transduction (*6, 7*). In this situation, after potential fusion between target and fusion particles, which should still be mediated by CD4/CXCR-4 and HIV Env, the infection of 293T cells (*lacking* CD4/CXCR-4) and transduction of the luciferase gene by the fused particles should be mediated by VSV-G. As shown in Fig. 2B, this is, in fact, the case and mixtures of CD4/CXCR-4 target and Wt-Env/G fusion particles resulted in high luciferase activity in transduced 293T cell lysates. The VSV-G-mediated luciferase activities were, in fact, 20–50 times higher than those obtained with HIV Env (Fig. 2A), consistent with the superior ability of VSV-G to mediate retroviral transduction as described by others (*6, 7*). It was initially a surprising observation that mixtures of Wt-Env/G fusion particles, or Env^{Fus-}/G fusion particles, having incorporated HIV Env mutated within the gp41 fusion peptide, plus CD4 target particles having incorporated CD4 *alone,* still led to significant transduction of luciferase (Fig. 2B). The values obtained in several different experiments were in the range of 20–50% of the values obtained with CD4/CXCR-4 target particles plus Wt-Env/G fusion particles. In fact, all further mixtures of target and fusion particles (CXCR-4 target particles with CXCR-4 alone, Env^{Bind-}/G fusion particles with defective HIV Env mutated at the CD4 binding site (Fig. 2B), or

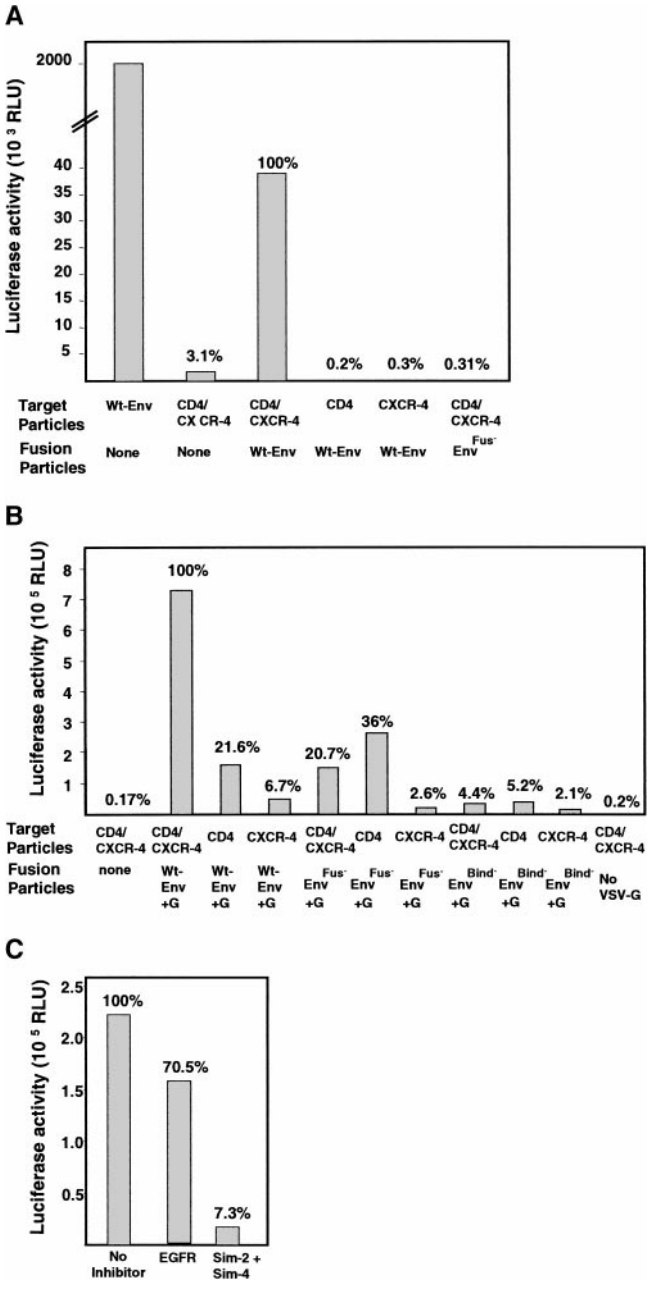

FIG. 2. Luciferase gene transduction after interviral particle fusion mediated by CD4/CXCR-4 and HIV Env. (A) HIV Env-mediated gene transduction. The glycoprotein compositions of the respective target and fusion particles, which were premixed prior to application to CD4/CXCR-4-expressing 293T cells, and the resulting luciferase activities (total activities per culture given on the y axis) in lysates of transduced cells are given for a representative experiment (from three independent experiments). The numbers above each block give the percentage luciferase activity in comparison to that generated by a mixture of CD4/CXCR-4 target particles and Wt-Env fusion particles. (B) VSV-G-mediated gene transduction. The glycoprotein compositions of the respective target and fusion particles, which were premixed prior to application to 293T cells (not expressing CD4/CXCR-4) and the resulting luciferase activities (total activities per culture given on the *y* axis) in lysates of transduced 293T cells are given for a representative experiment. G denotes VSV-G glycoprotein. The numbers above each block give the percentage luciferase activity in comparison to that generated by a mixture of CD4/CXCR-4 target particles and Wt-Env/G fusion particles. (C) Inhibition of transduction by monoclonal antibody against CD4. Mixtures of CD4/CXCR-4 target particles and Wt-Env/G fusion particles were either untreated (no inhibitor) or preincubated with irrelevant antibody (EGFR) or with antibody against CD4 (SIM-2 + SIM-4). The numbers above each block give the percentage luciferase activity in comparison to that of the untreated sample.

VSV-G alone in fusion particles (not shown)) also resulted in luciferase transduction as long as the fusion particles contained VSV-G glycoprotein. In these latter cases, the luciferase values obtained were in the range of 2–10% of the values obtained with CD4/CXCR-4 target particles plus Wt-Env/G fusion particles (Fig. 2B). On the other hand, when VSV-G was absent from fusion particles, and these were mixed with CD4/CXCR-4 target particles, only background levels of luciferase transduction into 293T cells were observed. We interpret these positive but clearly reduced luciferase transduction values, in the absence of CD4/CXCR-4/HIV-Env-mediated fusion, but in the presence of VSV-G, to be the result of particle fusion mediated by VSV-G on fusion particles and VSV-G cellular receptor which has been incorporated into target particles. This would be despite the fact that VSV-G normally requires low pH in the cellular endosome, after receptor-mediated uptake, to trigger membrane fusion. The VSV-G cellular receptor has been shown to include phospholipid components (*10, 11*), which would also be incorporated into virus-like particles. In those situations in which CD4 and HIV Env can bind to each other but not fuse (CD4 target particles, Env^{Fus-}/G fusion particles), the luciferase transduction values of mixed particles are significantly higher (20–50% of the values obtained with CD4/CXCR-4 target plus Wt-Env/G fusion particles) than in the absence of CD4/HIV Env binding (2–10%). This is presumably due to the binding between CD4 and HIV Env leading to a closer proximity between target and fusion particles and allowing more efficient membrane fusion mediated by VSV-G and its cellular receptor. Last, as a further proof of the role of CD4 in mediating binding and fusion between particles, the presence of CD4 monoclonal antibody during the incubation between CD4/CXCR-4 target particles and Wt-Env/G fusion particles very significantly inhibited luciferase transduction (Fig. 2C).

The results of this latter set of experiments confirm, on the one hand, the results obtained using the first strategy (Figs. 1A and 2A), namely that specific CD4/CXCR-4/HIV-Env-dependent interretroviral fusion can occur and that the resultant fused particles are infectious. The results, however, further indicate that, even in the absence of overexpression, cellular receptors for membrane viruses (VSV as an example) can be incorporated into foreign virus particles in amounts sufficient to mediate interparticle fusion with virus particles carrying the respective viral receptor. Thus, depending on the nature of the membrane virus, the tissue distribution of its cellular

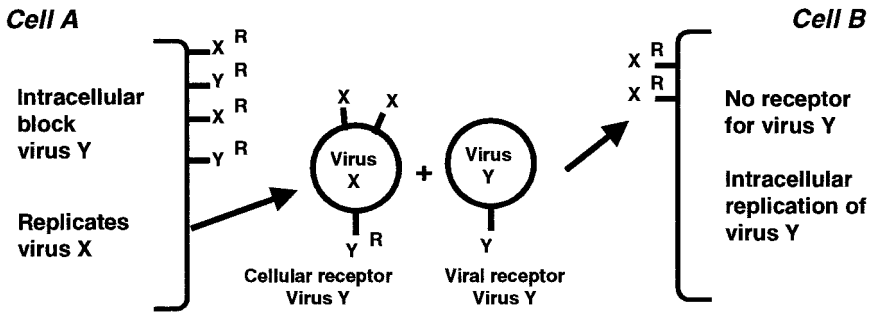

FIG. 3. Targeting of viral genome to cells lacking cellular receptor as a result of specific interviral fusion. The hypothetical producer cell, A, for virus X (left) carries cellular receptors for viruses X and Y (X^R and Y^R, respectively) but (in order to distinguish this phenomenon from pseudotyping) is unable to replicate virus Y. Released virus X, with incorporated Y^R, can fuse with virus Y, produced elsewhere in the infected organism. The resultant fused viral structure is able to transfer the genome of virus Y, via viral receptor X, to a novel target cell (right) lacking Y^R but carrying X^R.

receptor, and the availability of this molecule for incorporation into particles, interviral membrane fusion may be a previously unrecognized *in vivo* process (not achievable by simple pseudotyping) by which the genomes of membrane viruses can target cells lacking appropriate cellular receptor. A theoretical scenario describing such a situation is shown in Fig. 3. In this situation, membrane virus X, released from cells carrying cellular receptor for both virus X and virus Y (but intracellularly blocked for replication of virus Y) would incorporate cellular receptor for virus Y (Y^R) and thus fuse with virus Y itself (produced in permissive cells). The resulting fused viral structures would then be able to transfer the genome of virus Y to further cells lacking Y^R but carrying the cellular receptor for virus X (X^R). Depending on the viruses and target cells involved, this could have far-reaching consequences for the infected organism, e.g., stable production of virus Y within the animal from a hitherto unrecognized cell reservoir.

As already mentioned, based on the data reported by LaCasse *et al.* (*1*), the results obtained here in principle support the view that fixed preparations of well-defined, fusing CD4/CXCR-4 target and Wt-Env fusion particles may represent a potential HIV vaccine. In fact, the same strategy could be applicable to the glycoproteins of other pathogenic membrane viruses and their cellular receptors (e.g., hepatitis C virus). Further experiments will now aim, on the one hand, at optimizing fusion (e.g., by varying the particle concentration or the ratio of target and fusion particles). On the other hand, in order to analyze immune (and possibly protective) responses, preparations of noninfectious, subviral HIV-like target and fusion particles, paraformaldehyde fixed (at different time points) in the process of membrane fusion and concentrated by ultracentrifugation, should be inoculated into susceptible animal models.

Materials and Methods. Constructs employed. pNL4-3.Luc.R-E-, an *env*-defective HIV-1 proviral construct, encoding luciferase instead of *nef* (*8*), was used to generate target particles. PSG5-CD4-GpI was a kind gift from Marc Alizon (INSERM, Paris). It encodes GPI-anchored human CD4 (referred to in this paper as CD4) with the extracellular domain of CD4 and the C-terminal region of CD55. It was employed since, in our hands, this construct (plus CXCR-4) results in two to three times better infection of HIV-Env-expressing cells than Wt CD4 (data not shown). Expression of CXCR-4 was achieved employing pc.Fusin (*12*) (for target particles) or pBABE.Fusin (*12*) (to generate CD4/CXCR-4-expressing 293T cells, see below). The proviral construct, pNL4-3$\Delta\Psi\Delta$Env3, based on pNL4-3 (*13*), contains a frame-shift mutation at the beginning of the *env* gene (*3*) and a deletion in the packaging signal (*14*). It was employed to generate fusion particles. pLβAcenv/neo (referred to here as pHIV-Env) encodes Wt HIV Env (*15*). The derivatives referred to here as HIV-Env^{Fus-} and HIV-Env^{Bind-} carry mutations within the fusion peptide (gp41.2) (*16*) and CD4 binding site (*17*), respectively. pEx-VSV-G encodes the vesicular stomatitis virus G glycoprotein.

Generation of Target and Fusion Particles. pNL4-3.Luc.R-E-, plus PSG5-CD4-GpI and pc.Fusin singly and in combination (to generate target particles), or pNL4-3$\Delta\Psi\Delta$Env3 plus Wt or mutated versions of pHIV-Env in the presence or absence of pEx-VSV-G (to generate fusion particles), were cotransfected into 293T cells using standard calcium phosphate procedures. Forty-eight hours posttransfection, the amounts of released particles were quantitated by ELISA for HIV-1-CA (Innogenetics, Belgium). Expression of CD4, CXCR-4 (employing CXCR-4 monoclonal antibody (12G5) (*18*)), HIV Env, and VSV-G (antiserum from Lee Biomolecular, San Diego) were confirmed by indirect immunofluorescence analyses employing respective specific antisera as previously described (*9, 15*) (data not shown). Appropriate volumes of culture supernatants, filtered through a 0.45-μm filter, and representing equal amounts of different target particles on the one hand and equal amounts of different fusion particles on the other hand were adjusted to 8 μM Polybrene, mixed (at a ratio of 1:1) in a final volume of 2 ml and incubated for 90 min at 37°C, 5% CO_2, before application to subconfluent cultures of fresh indicator cells (either 293T cells or CD4/CXCR-4-expressing 293T

cells) in a 12-well (4.9 cm^2) multidish. CD4/CXCR-4-expressing 293T indicator cells were generated by stable expression of CD4 (from pIGRE-CD4 (*9*)) plus CXCR-4 (from pBABE.fusin (*12*)) in 293T cells. Seventy-two hours later, cell lysates were prepared and total luciferase activities per culture measured using standard procedures.

Inhibition of Particle Fusion. Culture supernatants from SIM-2 plus SIM-4 (1:1) hybridoma cells (*19*), releasing antibodies reacting with CD4 and able to inhibit infection by pNL4-3 HIV, or from H-EGFR-RI hybridoma cells (*20*), releasing antibodies against human epidermal growth factor receptor, which are thus irrelevant in this context, were added to the mixtures of target and fusion particles during the incubation period before application to indicator cells. The final concentration of mouse antibody was approx 1 μg/ml.

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Note added in proof. Alternative interpretations of the data presented could be (1) fusion particles independently fuse with target cells, thus depositing HIV Env or VSV-G on the cell surface, which now serve as receptors for the respective cellular receptors on target particles. This is unlikely in the case of VSV-G, which fuses with, and would be deposited on, the endosomal membrane. (2) Fusion between the membranes of target and fusion particles may not reach completion in suspension outwith the cell. Alternatively, preformed complexes between fusion-competent target and fusion particles, which have not yet completed fusion, may initially fuse with the target cell membrane, mediated by HIV Env or VSV-G (in this latter case, fusion would be in the endosome), and subsequently, fusion with the target particle membrane would go to completion. The data do not support or rule out this possibility. However, this consideration does not detract from the implications concerning host-range extension and potential application as vaccine strategy.

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